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US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDR-C
3100 Ricketts Point Road
Aberdeen Proving Ground, MD
21010-5400

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Cytokine regulation by MAPK activated kinase 2 in keratinocytes exposed to sulfur mustard ☆,☆☆

E. Chepchumba K. Yego, James F. Dillman III *

Cell and Molecular Biology Branch, US Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, 21010 MD, United States

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ABSTRACT

Uncontrolled inflammation contributes to cutaneous damage following exposure to the warfare agent bis(2-chloroethyl) sulfide (sulfur mustard, SM). Activation of the p38 mitogen activated protein kinase (MAPK) precedes SM-induced cytokine secretion in normal human epidermal keratinocytes (NHEKs). This study examined the role of p38-regulated MAPK activated kinase 2 (MK2) during this process. Time course analysis studies using NHEK cells exposed to 200 μM SM demonstrated rapid MK2 activation via phosphorylation that occurred within 15 min. p38 activation was necessary for MK2 phosphorylation as determined by studies using the p38 inhibitor SB203580. To compare the role of p38 and MK2 during SM-induced cytokine secretion, small interfering RNA (siRNA) targeting these proteins was utilized. TNF-α, IL-1β, IL-6 and IL-8 secretion was evaluated 24 h postexposure, while mRNA changes were quantified after 8 h. TNF-α, IL-6 and IL-8 up regulation at the protein and mRNA level was observed following SM exposure. IL-1β secretion was also elevated despite unchanged mRNA levels. p38 knockdown reduced SM-induced secretion of all the cytokines examined, whereas significant reduction in SM-induced cytokine secretion was only observed with TNF-α and IL-6 following MK2 knockdown. Our observations demonstrate potential activation of other p38 targets in addition to MK2 during SM-induced cytokine secretion.

1. Introduction

The chemical warfare agent sulfur mustard (bis(2-chloroethyl) sulfide; SM) is a bifunctional alkylating agent that leads to cutaneous damage. Changes that characterize skin damage due to SM include erythema, itching and blister formation (Papirmeister et al., 1991). These symptomatic changes occur following a latent period of several hours, and various underlying cellular events, for example, cell death and inflammation, are responsible for observable changes. Some SM-induced pro-inflammatory processes that have been documented are leukocyte infiltration (Dannenberg et al., 1985; Lindsay and Rice, 1996; Wormser et al., 2005), the release of inflammatory mediators such as prostaglandins and arachidonic acid (Lefkowitz and Smith, 2002; Dachir et al., 2004) and cytokine secretion (Sabourin et al., 2000). The skin provides a protective barrier from the environment, and keratinocytes are the predominant cell type in this tissue; they constitute 90% of this organ (Eckert, 1989). These cells secrete cytokines such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-1β (IL-1β) following exposure to SM (Arroyo et al., 2000). This cytokine secretion is regulated by the mitogen activated protein kinase p38 as demonstrated by studies using the p38 inhibitor SB203580 (Dillman et al., 2004). Reduced SM-induced secretion of the cytokines TNF-α, IL-6, IL-8 and IL-1β was observed when keratinocytes were incubated with this selective p38 inhibitor, which inactivates all p38 isoforms (α,β,γ and δ) by interacting with the ATP binding pocket. A similar observation was made when NHEKs were exposed to SM following p38-α knockdown using siRNA (Ruff and Dillman, 2010).

Abbreviations: SM, sulfur mustard; NHEK, normal human epidermal keratinocytes; MAPK, mitogen activated protein kinase; siRNA, small interfering RNA; MK2/MAPKAPK2, MAPK activated kinase 2; MK3/MAPKAPK3, MAPK activated kinase 3; MK5/MAPKAPK5, MAPK activated kinase 5; TNF-α, tumor necrosis alpha-α; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; LPS, lipopolysaccharide; HSP27, heat shock protein 27; TTP, tristetraprolin; HBSS, Hanks' balanced salt solution; qPCR, quantitative polymerase chain reaction; ERK, extracellular signal regulated kinase; JNK, c-jun N-terminal kinase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase- 2; ARE, adenosine and uridine rich element; 3'-UTR, 3′ untranslated region; GM-CSF, granulocyte–macrophage colony stimulating factor; G-CSF, granulocyte-colony stimulating factor; BRF1, butyrate response factor-1; AUF1, ARE/poly(U)-binding/degradation factor 1; miRNA, microRNA.

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* Corresponding author. Tel.: +1 410 436 1723; fax: +1 410 436 4150.
E-mail addresses: echepchumba.k.yego.ctr@us.army.mil (E. Chepchumba K. Yego), james.f.dillman2.civ@mail.mil (J.F. Dillman III).

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MAPK activated kinase 2 (MAPKAPK2/ MK2) is a downstream target of the p38 signaling cascade (Rouse et al., 1994; Gaestel, 2006). This 50 kDa serine/threonine kinase is regulated by p38 via phosphorylation at residues T25, T222, T272 and T334 (Ben-Levy et al., 1995). Phosphorylation at residue T334 is necessary for substrate binding and subsequent target phosphorylation (Engel et al., 1998; Meng et al., 2002; Gaestel, 2006). Structurally, MK2 contains a proline-rich N terminal, catalytic domain, and a C-terminal region containing a MAP kinase binding site (Engel et al., 1993; Zu et al., 1994, 1995; Meng et al., 2002). Two MK2 isoforms have been described in the literature, and the shorter isoform has a truncated C terminal domain (Stokoe et al., 1993; Cano et al., 1996).

MK2 controls several cellular processes such as cell stress, inflammation, cytoskeletal remodeling, cell division, proliferation and wound healing (Gaestel, 2006). Downstream targets that are regulated by MK2 via phosphorylation include heat shock protein 27 (HSP27), tristetraprolin (TTP), and Cdc25B1 (Gaestel, 2006). MK2 is structurally related to MAPK activated kinase 3 (MAPKAPK3/MK3) and MAPK activated kinase 5 (MAPKAPK5/MK5/PRAK). Studies using MK2, MK3 and MK5 knockout mice have greatly expanded our current understanding of MK signaling during the development of various pathological conditions (Kotlyarov et al., 1999; Shi et al., 2003; Ronkina et al., 2007). Under basal conditions, these mice display normal phenotype and reproductive patterns. Lipopolysaccharide (LPS) stimulation induces endotoxic shock in MK3 and MK5 knockout mice, whereas MK2 knockout mice are protected from this effect, demonstrating a distinctive role for this specific MAPK activated kinase during inflammation (Kotlyarov et al., 1999; Shi et al., 2003; Gaestel, 2006; Ronkina et al., 2007). Impaired TNF-α and IL-6 secretion contributes to this diminished inflammatory response in MK2 knockout mice (Kotlyarov et al., 1999). Even though serum TNF-α levels are reduced in the knockout mice, TNF-α mRNA levels in wild-type and MK2 knockout mice are comparable. This indicates that cytokine regulation by MK2 primarily occurs at the posttranscriptional level.

Selective p38 inhibitors such as SB203580 have successfully been used for the study of this kinase. However, side effects such as liver toxicity and off target effects have restricted the effective use of p38 inhibitors in a clinical setting (Coulthard et al., 2009). Downstream p38 signaling molecules such as MK2 have been put forth as alternative therapeutic targets, and current research on this molecule has demonstrated therapeutic potential for agents that interfere with this kinase (Mourey et al., 2010). Uncontrolled activation of this kinase in epidermal tissue is also associated with inflammatory skin conditions such as psoriasis and contact dermatitis (Johansen et al., 2006; Funding et al., 2009). For these reasons, we examined the role of MK2 during SM-induced keratinocyte cytokine secretion. The effect of SM on MK2 activation and the necessity for this kinase during cytokine secretion were evaluated.

2. Materials and methods

2.1. Keratinocyte cell culture

Normal human epidermal keratinocytes (NHEKs) were obtained from Lonza (Walkersville, MD) and maintained in Keratinocyte Gold Cell Medium (KGM Gold™, Lonza) at 37 °C with 5% CO_2 in a humidified incubator. For experimentation, cells were plated at a density of 1.0×10^3 cells/cm^2 in T25 flasks, and experiments were initiated when cells were approximately 70–80% confluent. All the NHEK experiments utilized cells at passage 2 or 3 postcryopreservation.

2.2. SM agent exposure

A frozen aliquot of neat SM (Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD) in KGM Gold was thawed and vortexed to generate a 4 mM stock solution that was immediately used for exposure. SM purity was ⩾95% as determined by freezing point depression, ^{1}H NMR, ^{12}C NMR, ^{31}P NMR, GC/TCD, GC/MSD, and/or acid–base titration methods. Agent exposure using 200 μM SM was performed as previously described (Ruff and Dillman, 2010). Briefly, cells were exposed to SM and samples were processed for analysis 5, 15, 30 and 480 min (Western blot analysis and real time quantitative PCR [qPCR]) or 24 h (cytokine analysis) after exposure. Parallel time-matched unexposed controls were included with each group. Immediately after SM treatment, cells were incubated in a chemical surety fume hood for 30 min at 37 °C with ambient air, and then placed at 37 °C with 5% CO_2 for the remainder of the exposure. A naïve control that was not subjected to chemical surety fume hood incubation was included with all the experiments. To inhibit p38 kinase, cells were pre-treated with 10 μM SB203580 (Tocris, Ellisville, MO) for 1 h before SM exposure. Pre-treatment conditions were based on previous studies (Dillman et al., 2004), and the inhibitor was retained in the media during SM exposure. Control cells received DMSO vehicle (1 μl/ml).

2.3. Protein isolation and quantification

Whole cell protein lysates were generated from treated cells as previously described (Ruff and Dillman, 2010). Cell culture media was removed, and cells were rinsed twice with Hanks' balanced salt solution (HBSS). Cells were then lysed with 400 μL sodium dodecyl sulfate (SDS) lysis buffer (125 mM Tris, 4% SDS, 20% glycerol, and pH 6.8) and homogenized at 4 °C using a Mini-Bead Beater-96 (BioSpec Products Inc., Bartlesville, OK) for 30 s. Total protein was quantified using the bicinchoninic protein assay (Pierce Endogen, Rockford, IL).

2.4. Western blot analysis

Whole cell protein lysates generated as described above were prepared for electrophoresis. XT sample lysis buffer (BioRad, Hercules, CA) and 1X reducing buffer (BioRad) were added to 30 μg protein. Samples were then heated for 5 min at 100 °C and resolved using SDS polyacrylamide gel electrophoresis with 10% Bis–Tris polyacyrlamide gels (BioRad) and MOPS buffer, pH 7.0 (BioRad). Constant voltage of 187 V at room temperature was maintained during the 1 h electrophoresis. Resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membrane using the I-Blot transfer system (Invitrogen, Carlsbad, CA) and blocked using Superblock T20 blocking buffer (Thermo Scientific, Waltham, MA). All primary antibodies used for this study were obtained from Cell Signaling Technology (Danvers, MA). Rabbit antibody against phospho (thr 334) MK2 (Cat#3007, 1:750 dilution) and primary rabbit antibody against phospho (tyr 180/thr 182) p38 (Cat#9215, 1:1000 dilution) were used to probe for phosphorylated proteins. Horse-radish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody from Invitrogen (1:3000 dilution) and ECF reagent (GE Healthcare, Piscataway, NJ) were used for protein detection. A Typhoon Trio + scanner and Image Quant software, Version 6.0 (GE Healthcare), was used to visualize and quantify Western blot bands. Membranes were stripped using a low pH glycine solution (0.4 M glycine, 0.1% SDS, 1% Tween20, and pH 2.2) for 1 h at room temperature and blocked using Superblock T20 blocking buffer. Stripped membranes were re-probed against total amounts of respective proteins and actin using rabbit antibody against total MK2 (Cat#3042, 1:1000 dilution), rabbit antibody against total p38 (Cat#9212, 1:1000), and rabbit antibody against actin (Cat#4970, 1:5000). Band intensities were quantified, normalized to actin, and graphed as mean ± SEM relative to naïve control group.

2.5. Quantitative real time PCR analysis

Total RNA was isolated from treated NHEK cells using Qiagen RNeasy mini kit (Qiagen, Valencia, CA) with DNase digestion according to manufacturer's instructions. RNA quality and quantity were determined using an Agilent bioanalyzer (Agilent Technologies, Santa Clara, CA) and NanoDrop ND-1000 UV–vis 186 spectrophotometer (Thermo Scientific) respectively.

Reverse transcription was performed using 2 μg of input RNA with an Applied Biosystems high capacity cDNA reverse transcription kit (Foster City, CA). To determine relative mRNA changes, cDNA was subjected to real time quantitative PCR (qPCR) analysis using the TaqMan™ gene expression assay system (Applied Biosystems) and the Applied Biosystems 7500 real-time PCR detection system. The following targets were assessed: human MK2 (assay ID Hs00358962_m1), human TNF-α (assay ID Hs00174128_m1), human IL-1β (assay ID Hs01555410_m1), human IL-6 (assay ID Hs00985639_m1) and human IL-8 (assay ID Hs00174103_m1). Assays were performed in triplicate, and 18S ribosomal RNA (Applied Biosystems Cat# 4319413E) was used to normalize for the starting amount of cDNA. ΔC_t values were determined using values from the 18S ribosomal RNA internal reference gene. The $\Delta\Delta C_t$ values relative to the naïve control were then determined, and fold changes relative to this control were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. siRNA transfection

siRNA transfection was performed using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA) as previously described (Ruff and Dillman, 2010) for the knockdown of target genes MK2 and p38 MAPK. Briefly, siRNA was diluted in 500 μl OPTI-MEM reduced serum media (Invitrogen), and in a separate tube, transfection reagent (1.5 times the volume of siRNA) was also diluted in 500 μl OPTI-MEM reduced serum media. Lipofectamine and siRNA-containing solutions were then mixed and incubated for 15 min at room temperature before addition to NHEK cells in tissue culture flask. Lipofectamine-containing media was replaced with fresh KGM Gold media after 5 h. Knockdown efficiency at the protein level was evaluated 24 h after transfection using Western blot analysis. mRNA levels were evaluated after 18 h using real time qPCR. SM treatments were initiated 24 h after transfection. Two independent Validated Stealth siRNA duplexes (Invitrogen) that recognize different regions on MK2 mRNA were utilized for MK2 knockdown (Table 1). p38 Validated Stealth siRNA was utilized for p38 knockdown (Table 1). Validated Stealth non-targeting siRNA was included with all experiments to control for off target effects (Invitrogen Cat# 45-2002).

2.7. Cytokine analysis

To determine amounts of cytokine (TNF-α, IL-1β, IL-6 and IL-8) secreted into cell culture medium, multiplex cytokine analysis was performed using the Milliplex MAP kit (Millipore, Billerica, MA) and Bio-Plex system array reader (BioRad). Assay was performed according to manufacturer's specification. Briefly, cell culture medium (25 μL) was incubated overnight at 4 °C in a 96-well plate with pre-mixed bead sets (25 μl bead slurry) for the analytes of interest (TNF-α, IL-1β ,IL-6 and IL-8). Following overnight incubation, plates were washed and incubated with biotinylated detection antibody for 1 h at room temperature. Plates were then incubated with the reporter molecule, streptavidin phycoerythrin-conjugate. The Bio-Plex system array reader with Bio-Plex Manager 5.0 software (BioRad) was used for sample quantification.

Table 1
siRNA duplexes and the complementary sequence on the target transcript.

siRNA	Target sequence on transcript
MK2 Duplex 1 (Oligo ID VHS40763)	CCAGUAUCUGCAUUCAAUCAACAUU
MK2 Duplex 2 (Oligo ID VHS40764)	CCAGUAUGAAUUUCCCAACCCAGAA
p38 Duplex (Oligo ID VHS40416)	AUACUUUAGACCUCGGAGAAUUUGG

2.8. Statistical analysis

Time course data were analyzed for significant difference using two way analysis of variance (ANOVA) with post hoc analysis using Bonferroni's test to determine changes among groups. All other data was analyzed using one way ANOVA with post hoc analysis using Dunnett's multiple comparison test with the exception of IL-1β cytokine data, which was analyzed using Kruskal–Wallis test with post hoc analysis using Dunn's test since this data set was not normally distributed as determined by a frequency histogram. All other data was both quantitative and normally distributed.

3. Results

3.1. SM-induced MK2 phosphorylation in keratinocytes occurs in a time-dependent manner

Activation of the p38 signaling protein MK2 was evaluated as demonstrated by phospho (Thr 334) MK2 Western blot analysis. Exposing NHEK cells to SM (200 μM) increased MK2 phosphorylation with significant changes occurring at 15 min (4.7 ± 0.9-fold increase) postexposure (Fig. 1A). Elevated MK2 phosphorylation was sustained at 30 min (5.5 ± 2.2-fold increase) and 480 min/8 h (6.7 ± 2.7-fold increase) postexposure. Similar increases were observed when data was normalized to total MK2 (data not shown). Multiple bands were observed on the phospho-MK2 Western blots. This potentially represents multiple phosphorylation states of the protein since there are several phosphorylation targets on MK2 (Ben-Levy et al., 1995). Alternatively the lower band may represent the truncated MK2 isoform (Cano et al., 1996). The higher 49 kDa band was used for densitometry analysis since total MK2 Western blot detects this band and this is the predicted molecular weight for phosphor (Thr 334) MK2.

3.2. p38 kinase activity is necessary of SM-induced MK2 phosphorylation

To establish the necessity for p38 activity during MK2 activation following exposure to SM in keratinocytes, MK2 phosphorylation was evaluated following keratinocyte pre-treatment with the selective p38 inhibitor SB203580 (10 μM) for 1 h. Interfering with p38 kinase activity prevented SM-induced MK2 phosphorylation at all the time points examined, demonstrating the dependence of MK2 function on signaling from this upstream kinase (Fig. 2). p38 inhibition decreased SM-induced MK2 activation by 88.6 ± 12.7% at 30 min postexposure and 87.7 ± 10.1% at 480 min postexposure (Fig. 2).

3.3. MK2 siRNA efficiently knocks down MK2

MK2 knockdown using siRNA was evaluated at the protein and message level using Western blot analysis and real time qPCR respectively. NHEK cells were transfected with MK2 siRNA, using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions as described above in Section 2. Two independent duplexes were utilized (Table 1). Non-targeting siRNA was also included with the experiments to control for off target effects. The potential for MK2 siRNA to knockdown protein levels was

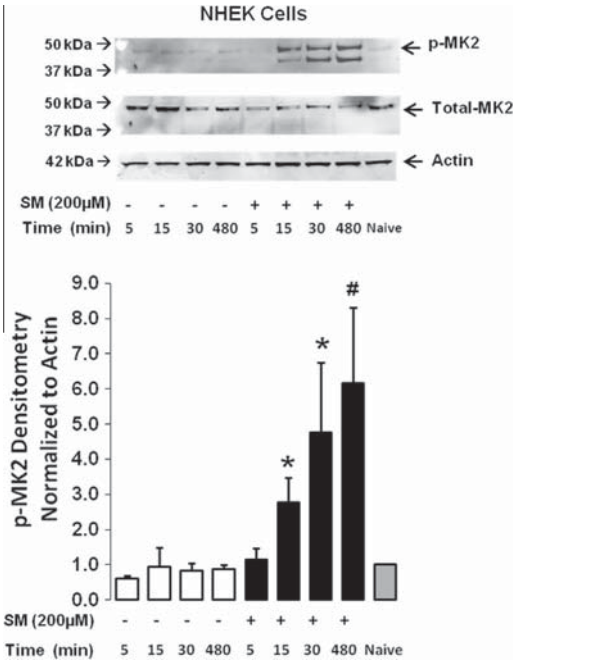

Fig. 1. SM induces MK2 phosphorylation in keratinocytes. Primary NHEK cells were exposed to SM (200 μM) and samples processed at 5, 15, 30 or 480 min post exposure. Corresponding time-matched control samples were left unexposed. Whole cell lysates were generated from treated cells and subjected to Western blot analysis to detect MK2 activation via phosphorylation at residue 334. Membranes were stripped and re-probed for total MK2 and actin. Phospho-MK2 (p-MK2) band intensities were quantified using densitometry analysis and normalized to actin; average values from independent biological replicates were determined. Two distinct bands were detected during phospho MK2 analysis, possibly representing two different MK2 isoforms (Cano et al., 1996) or multiple phosphorylation states (Ben-Levy et al., 1995). MK2 densitometry analysis was performed using the higher molecular weight (49 kDa) band. Values are graphed relative to naïve value ± SEM ($n = 4$ for A, $n = 3$ for B, $^{*}p < 0.05$ and $^{\#}p < 0.001$ compared to −SM condition at corresponding time point).

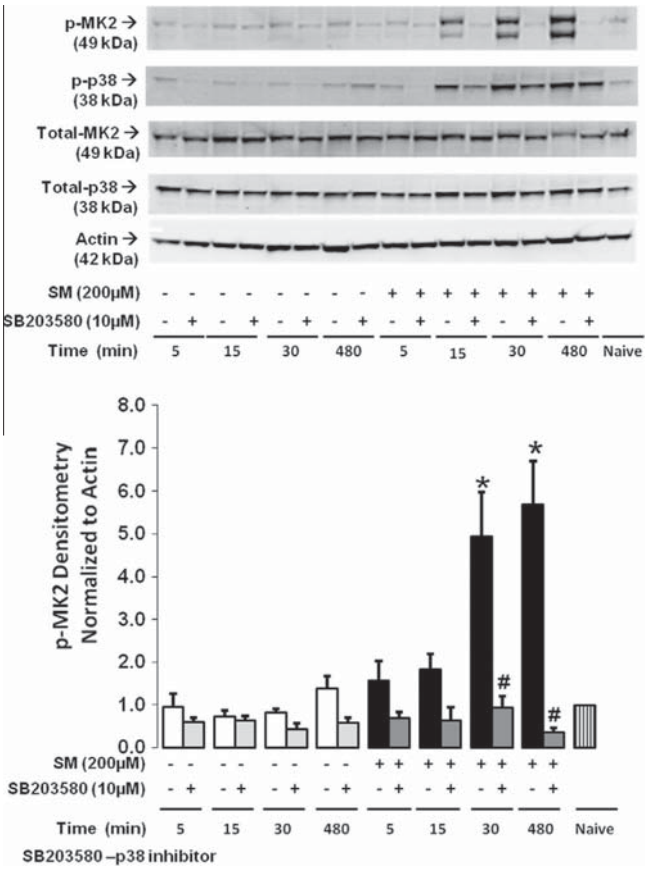

Fig. 2. p38 inhibition prevents SM-induced MK2 phosphorylation in keratinocytes. Primary NHEK cells were pre-treated with the p38 inhibitor SB203580 (10 μM) or control DMSO (1 μl/ml) for one hour then exposed to SM (200 μM). Samples were processed 5, 15, 30 or 480 min after SM exposure. Corresponding control samples were left unexposed. Whole cell lysates were generated from treated cells and subjected to Western blot analysis to detect MK2 activation via phosphorylation as described in Fig. 1. p38 activation was also monitored using Western blot analysis. Data are expressed relative to naïve sample ±SEM ($n = 4$, $^{*}p < 0.05$ compared to −SM and −SB203580 condition at corresponding time point, $^{\#}p < 0.001$ compared to +SM and −SB203580 at corresponding time point).

determined for four different siRNA concentrations: 1, 2, 4 and 6 pmol/cm^2. Protein knockdown of 49.5 ± 10.6% and 56.1 ± 7.7% was obtained with MK2 duplex-1 siRNA and MK2 duplex-2 siRNA respectively at 4 pmol/cm^2 (Fig. 3A). Similar knockdown of 49.2 ± 11.1% and 64.1 ± 6.2% was obtained with MK2 duplex-1 siRNA and MK2 duplex-2 siRNA respectively at 6 pmol/cm^2 at 24 h posttransfection (Fig. 3A). Additional evaluation of these MK2 duplexes under these conditions confirmed diminished MK2 message levels at 18 h posttransfection with knockdown efficiencies that were greater than 72% at 4 and 6 pmol/cm^2 for both MK2 siRNA duplexes (Fig. 3B). However, increasing siRNA concentration beyond 6 pmol/cm^2 with MK2 duplex-2 augmented SM-induced IL-6 and IL-8 secretion despite protein knockdown (data not shown). Therefore, all subsequent studies utilized 4 pmol/cm^2. All experiments were also performed between 24 and 48 h since knockdown using both siRNAs was sustained at 48 h, but MK2 was re-expressed at 72 h (data not shown).

3.4. Effect of MK2 and p38 knockdown on SM-induced keratinocyte cytokine secretion

To compare the role of MK2 and p38 during SM-induced inflammation, cytokine amounts in the cell culture media were quantified using multi-plex cytokine analysis following protein knockdown and SM (200 μM) treatment for 24 h. Cytokines that were analyzed are TNF-α, IL-1β, IL-6 and IL-8. For all the transfection conditions assessed, SM-induced cytokine secretion was significantly higher compared to corresponding unexposed control (Fig. 4). Nevertheless, levels of SM-induced cytokine secretion from cells transfected with p38 siRNA were lower than in control siRNA-transfected cells under the same conditions. Cytokine secretion was reduced by 43.2 ± 5.5% for TNF-α, 66.3 ± 6.8% for IL-1β, 52.1 ± 6.1% for IL-6 and 44.0 ± 8.9% for IL-8. The efficiency of p38 knockdown in reducing SM-induced cytokine secretion was higher than that of MK2 knockdown. Significant decreases in cytokine secretion were observed with TNF-α (32.9.0 ± 12.2%) and IL-6 (35.1 ± 7.1%) following knockdown with MK2 siRNA duplex-2 and MK2 siRNA duplex-1 respectively. MK2 knockdown induced a minor reduction in SM-induced IL-8 secretion that was not statistically significant. In addition, these same conditions had no significant effects on SM-induced IL-1β secretion.

3.5. Effect of MK2 and p38 knockdown on cytokine mRNA levels following SM exposure

p38 and MK2 modulate cytokines at both the transcriptional and posttranscriptional level (Heidenreich et al., 1999; Kotlyarov et al., 1999; Ronkina et al., 2007). Experimental conditions that have no effect on transcript levels despite changes in cytokine secretion are reflective of control primarily at the posttranscriptional level. To determine the role of p38 and MK2 at the posttranscriptional level, the effect of knocking down these proteins on

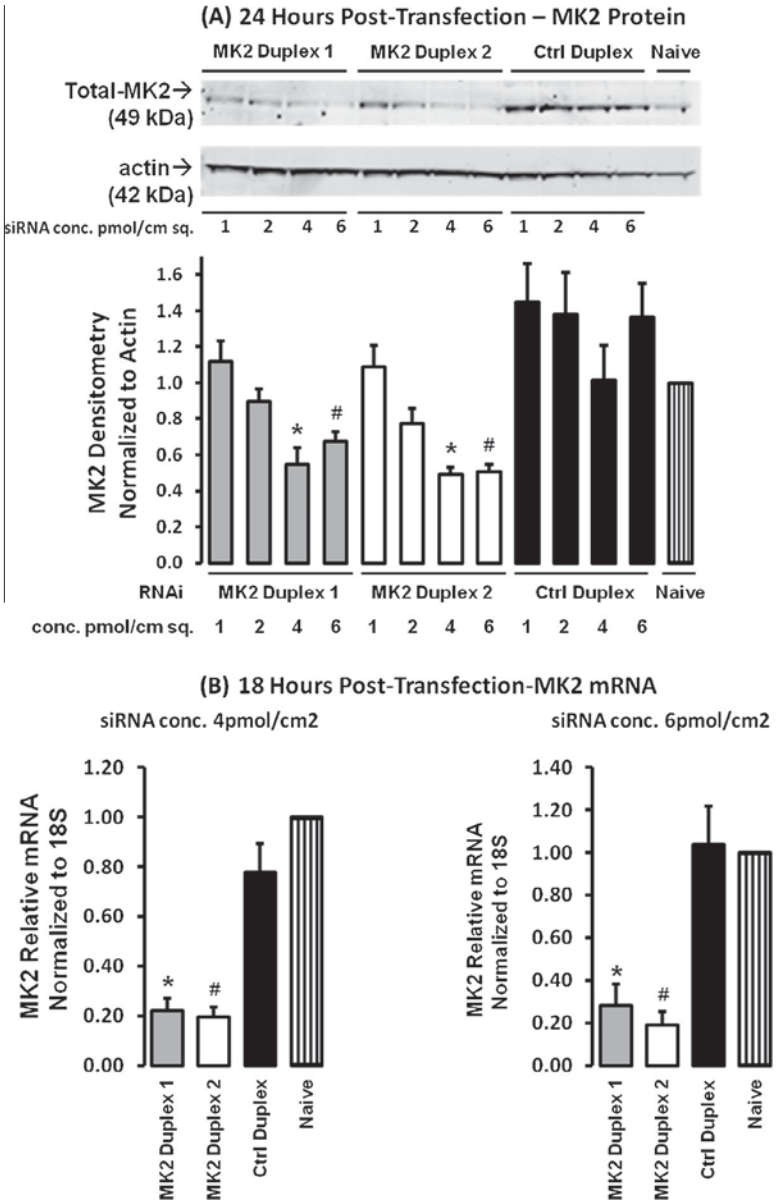

Fig. 3. MK2 siRNA efficiently knocks down MK2 in keratinocytes. NHEK cells were transfected with MK2 siRNA duplex 1, MK2 siRNA duplex 2 or control siRNA at various concentrations (1, 2, 4 or 6 pmol/cm^2). (A) To determine the efficiency of MK2 protein knockdown, whole cell lysates generated from transfected cells at 24 h posttransfection were subjected to total MK2 Western blot analysis. Membranes were stripped and re-probed for actin. MK2 band intensities were quantified using densitometry analysis and normalized to actin; average values from independent biological replicates were determined. Data are expressed relative to naïve sample ± SEM (n = 5, * p < 0.05 compared to 4 pmol/cm^{2}control siRNA, $^\#$ p < 0.05 compared to 6 pmol/ cm^2 control siRNA). (B) NHEK cells were transfected with siRNA (4 or 6 pmol/cm^2) as described above, and total RNA was isolated 18 h after transfection. MK2 mRNA levels quantified using real time qPCR were normalized to 18S ribosomal RNA, and fold changes relative to naïve control were determined. Independent experiments are graphed as means ±SEM (n = 5, * p < 0.05 compared to 4 pmol/cm^{2}control siRNA, $^\#$ p < 0.05 compared to 6 pmol/cm^2 control siRNA).

cytokine mRNA levels following NHEK exposure to SM was assessed. Real time qPCR was used to quantify relative mRNA levels for the cytokines TNF-α, IL-1β, IL-6 and IL-8, at 8 h after SM exposure, following NHEK transfection with control siRNA, MK2 siRNA or p38 siRNA (4 pmol/cm^2). Results from IL-1β analysis indicate that SM has no effect on mRNA levels even though increased secretion of this cytokine occurs following SM exposure (Fig. 5). With the exception of IL-1β mRNA, expression levels for all the other cytokines examined were significantly higher with SM-exposed cells compared to corresponding unexposed control for both control and target siRNA-transfected cells. Nevertheless, p38 knockdown significantly reduced cytokine mRNA levels compared to control siRNA-transfected cells by 45.1 ± 15.5% for TNF-α, 63.4 ± 11.5% for IL-6 and 57.6 ± 8.4% for IL-8. Similar to protein analysis, the potential for p38 siRNA to reduce SM-induced cytokine up regulation at the mRNA levels for TNF-α, IL-6 and IL-8 was higher compared to that of MK2 siRNA. MK2 knockdown using MK2 siRNA duplex 1 significantly reduced SM-induced expression of TNF-α by 34.0 ± 9.2%.

4. Discussion

Excessive inflammation plays a central role during SM-induced cutaneous damage (Ghabili et al., 2010; Shakarjian et al., 2010), and our studies evaluated the role of the p38-regulated kinase MK2 during SM-induced keratinocyte secretion of pro-inflammatory cytokines. The studies presented in this manuscript demonstrate that SM activates MK2 via phosphorylation at residue 334 in keratinocytes as determined using a phospho-specific antibody.

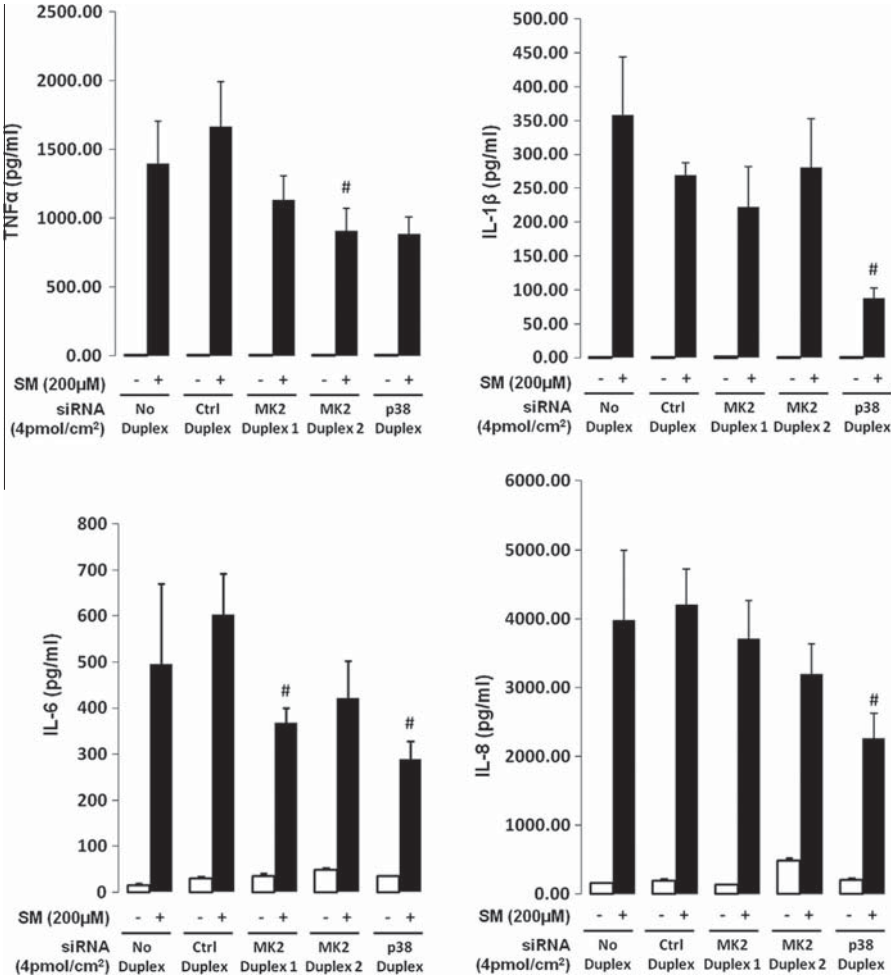

Fig. 4. Effect of MK2 and p38 knockdown on SM-induced secretion of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-8. NHEK cells were transfected with control siRNA, MK2 siRNA duplex 1, MK2 siRNA duplex 2, p38 siRNA (4 pmol/cm^2). Control group with no transfection was also included with the study. Cells were exposed to SM (200 μM) 24 h after transfection. Control cells were left unexposed. Multiplex cytokine analysis was performed to determine TNF-α, IL-1β, IL-6 and IL-8 levels in the media 24 h after exposure to SM. Data presented represent average values from independent biological replicate ± SEM ($n = 8$ for control and MK2 knockdown, $n = 6$ for p38 knockdown, $^{\#}$ $p < 0.05$ compared to control siRNA + SM).

Increased MK2 phosphorylation was observed beginning at 15 min postexposure and sustained for 8 h. This rapid activation is dependent on SM-induced p38 signaling. MK2 knockdown reduced SM-induced secretion of TNF-α and IL-6, demonstrating a role for this kinase during SM-induced inflammation. Nonetheless, the efficiency of SM-induced cytokine inhibition following p38 knockdown is higher compared to that of MK2 knockdown, thus demonstrating potential activation of additional p38-regulated pathways. Mitogen and stress-activated protein kinase 1 (MSK1) is an alternative p38 target that may regulate cytokines (Funding et al., 2006; Kjellerup et al., 2008), and MSK1 activation in mouse keratinocytes exposed to SM has previously been documented (Rebholz et al., 2008). Furthermore, studies using human keratinocytes and MSK1 siRNA have shown that increased TNF-α, IL-6 and IL-8 secretion following treatment with the p38 agonist anisomysin is dependent on MSK1 (Funding et al., 2006). MSK1 primarily regulates cytokines at the transcriptional level.

Keratinocyte cytokine mRNA expression profiles for the cytokines TNF-α, IL-6 and IL-8 correlated well with secretion profiles. On the other hand, changes in IL-1β mRNA levels following SM exposure and/or MK2 knockdown were not statistically significant despite increased cytokine secretion. This implicates translational or posttranslational processes. Molecular events that control cytokine secretion may also be involved. The pro-death and pro-inflammatory enzyme caspase-1/interleukin 1β converting enzyme is a well known regulator of IL-1β processing and secretion (Miller et al., 1997). This enzyme controls IL-1β secretion at the posttranslational level by cleaving inactive pro-IL-1β to generate the active form of this cytokine. No studies have examined the effects of SM on caspase-1. It would be interesting to determine whether interfering with this pro-death and pro-inflammatory enzyme protects cells from SM-induced damage.

Foundational work on MK2 using knockout mice implicates this kinase during posttranscriptional cytokine regulation (Kotlyarov et al., 1999). Even so, protein modulation by MK2 at the transcriptional level has also been documented (Heidenreich et al., 1999; Janknecht, 2001). The transcription factors serum response factor (SRF) and ER81 are regulated by MK2 through phosphorylation (Heidenreich et al., 1999; Janknecht, 2001). Our studies are consistent with a role for MK2 that modulates message levels for the targets TNF-α, IL-6 and IL-8. mRNA levels from SM-treated cells transfected with control siRNA and MK2 siRNA were not comparable, and SM-induced cytokine secretion profiles for these targets were similar to message profiles. On the other hand, these observations may reflect posttranscriptional cytokine control through mechanisms that involve mRNA destabilizing proteins (Buzby et al., 1999; Carballo et al., 2000; Stoecklin et al., 2002). ARE-associated RNA binding proteins decrease mRNA levels by destabilizing transcripts via mechanisms that involve exosome recruitment and increased exonuclease activity (Anderson, 2008). Interestingly, the

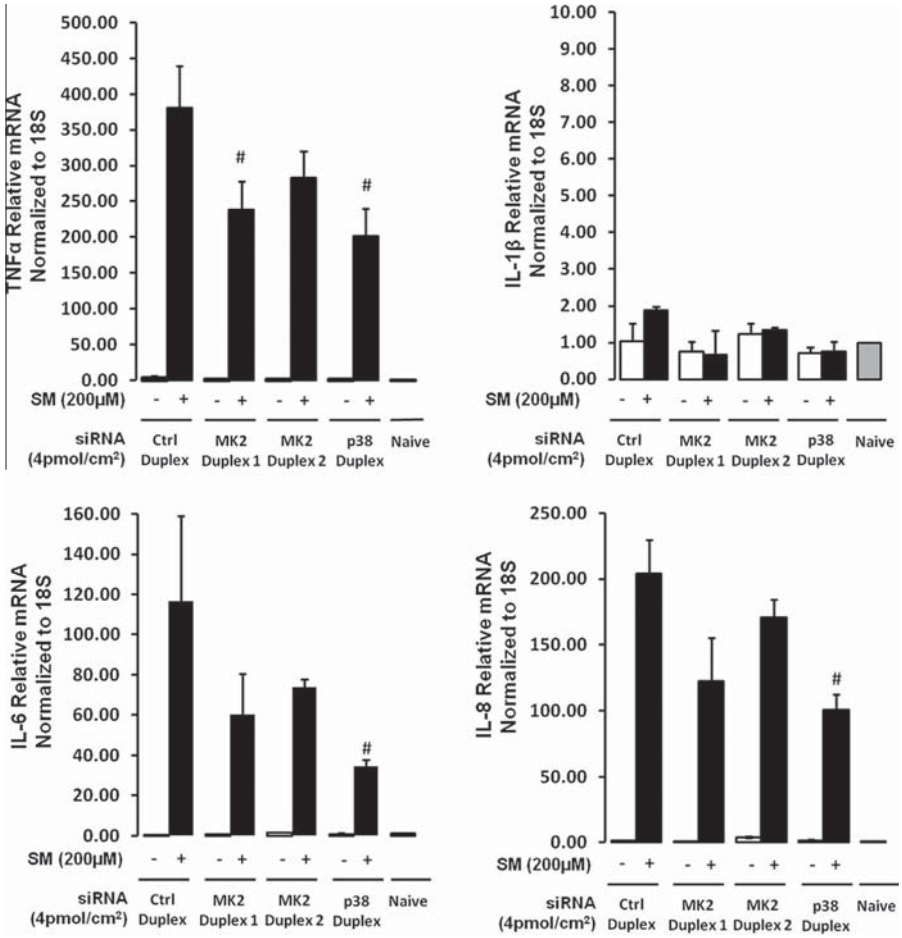

Fig. 5. Effect of MK2 and p38 knockdown on keratinocyte expression of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-8 following SM treatment. NHEK cells were transfected with MK2 siRNA duplex 1, MK2 siRNA duplex 2, p38 siRNA or control siRNA (4 pmol/cm^2). Cells were exposed to SM (200 μM) 24 h after transfection. Control cells were left unexposed. Total RNA was isolated from cells 8 h after exposure, and relative mRNA changes for the cytokines TNF-α, IL-1β, IL-6 and IL-8 were determined using quantitative real time PCR. mRNA levels were normalized to 18S RNA. Fold changes relative to naïve control were then established, and average values from independent biological replicates determined. Data represents mean ± SEM (n = 6, $^{\#}$ p < 0.05 compared to control siRNA + SM).

mRNA destabilizing protein TTP is a direct phosphorylation target of MK2 (Hitti et al., 2006; Katsanou et al., 2006; Sandler and Stoecklin, 2008). This protein promotes the degradation of several cytokines, including TNF-α, IL-6 and IL-8 mRNA (Anderson, 2008). TTP phosphorylation by MK2 interferes with TTP–mRNA binding and subsequent TTP-induced mRNA degradation, which in turn leads to elevated cytokine levels. Further studies on the role of RNA binding proteins on cytokine regulation during cutaneous damage by toxic agents such as sulfur mustard would greatly enhance our understanding of this process.

In addition to targeting inflammation, proposed countermeasures for the treatment of SM injuries modulate other biochemical and cellular events including cell death (Smith, 2009). Potential cross talk between inflammation and cell death may regulate skin damage since anti TNF-α antibodies reduce SM-induced cutaneous pathology and cell death (Wormser et al., 2005).

The p38 signaling pathway does not intrinsically control keratinocyte cell death due to SM exposure (Ruff and Dillman, 2010). Nonetheless, this pathway may participate during paracrine activation of this process since TNF-α cytokine secretion is controlled by p38 and TNF-signaling regulates SM-induced cutaneous damage (Wormser et al., 2005). Cell death via pyroptosis may also contribute to SM-induced cutaneous damage. This cell death mechanism resulting from inflammation is characterized by an augmented caspase-1-IL-1β signaling pathway. Robust IL-1β secretion occurs in SM-exposed keratinocytes.

The potential for therapeutic mechanisms that target MK2 for the treatment of pro-inflammatory conditions is promising (Schlapbach et al., 2008; Mourey et al., 2010). Observations from this study indicate that anti-inflammatory agents targeting MK2 as part of a combinatorial therapy may be useful for the control of pro-inflammatory cytokine secretion due to SM exposure. Other pro-inflammatory molecules that respond to SM exposure in keratinocytes or cutaneous tissue include extracellular signal regulated kinase (ERK) MAPK, c-jun N-terminal kinase (JNK) MAPK, NF-κB, endothelial Nitric Oxide Synthase (eNOS), inducible Nitric Oxide Synthase (iNOS), cyclooxygenase-2 (COX-2), prostaglandin E and the aforementioned MSK1 (Nyska et al., 2001; Dachir et al., 2004; Dillman et al., 2004; Minsavage and Dillman, 2007; Rebholz et al., 2008; Steinritz et al., 2009; Ruff and Dillman, 2010). Our studies are the first to report MK2 activation by SM. Therefore, the interaction between MK2 signaling and these other pathways is unknown. Nonetheless, cross talk between MK2 and the NF-κB pathway as well as COX-2 protein synthesis in other systems has previously been shown (Gorska et al., 2007; Streicher et al., 2010). Therefore, future studies will examine cross talk between the MK2 pathway and other pro-inflammatory signaling pathways and potential synergistic effects on cytokine secretion and inflammation.

Uncontrolled inflammation for most cutaneous conditions is associated with detrimental effects. However, MK2 activation and subsequent cytokine secretion are necessary for wound healing during the repair of mechanically injured mouse skin

(Thuraisingam et al., 2010). This observation was demonstrated by studies using MK2 knockout mice whereby there was a correlation between delayed wound healing and reduced secretion of the cytokines TNF-α, IL-6, IL-8, IL-1β, RANTES, GM-CSF and interferon-γ (IFN-γ). Most of these cytokines are regulated by MK2 following SM exposure in keratinocytes. Therefore, additional *in vivo* studies using MK2 knockout mice to assess the effect of MK2 inhibition on other essential cellular processes such as wound healing should be performed. Limitations from the use of siRNA technology also warrant the need for these studies. Augmented IL-6 and IL-8 secretion was observed when higher concentrations (6 pmol/cm^2 and 10 pmol/cm^2) of MK2 duplex-2 were utilized for target knockdown. This potentially reflects a sequence-specific immunostimulatory effect since MK2 knockdown using a second siRNA that targets the same region had similar effects (data not shown). siRNA immunostimulation is a well documented effect of select siRNAs that may manifest under specific conditions such as higher siRNA concentrations (Robbins et al., 2009; Vickers et al., 2009; Jackson and Linsley, 2010). This effect is associated with secretion of these two cytokines *in vitro* and *in vivo* (Robbins et al., 2009). The necessity for *in vivo* MK2 analysis is further demonstrated by two studies on different acute contact dermatitis models which indicate that the protective effect of MK2 is stimulus specific (Funding et al., 2009; Schottelius et al., 2010). Knocking down MK2 was protective during oxazolone-induced cutaneous inflammation (Funding et al., 2009) but not 2,4-dinfluorobenzene (DNFB)-induced cutaneous inflammation (Schottelius et al., 2010). Follow up *in vivo* studies would establish the pros and cons of potential therapies that interfere with MK2 signaling for the treatment of SM-induced wounds.

Disclaimer

The views expressed in this manuscript are those of the authors and do not reflect the official policy of the Department of Army, Department of Defense, or the US Government.

Conflicts of Interest

There are no known conflicts of interests.

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